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Modification of the Carcinogenic Process in Colorectal Cancer by Endogenous and Exogenous Factors: Effect of Colestipol Hydrochloride on Tumors Induced by Dimethylhydrazine

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The effect of the bile acid sequestrant, colestipol hydrochloride, on the incidence of dimethylhydrazine-induced tumors of the large intestine was determined in male Swiss mice. The subcutaneous administration of dimethylhydrazine (15 mg/kg) produced tumors in approximately 89% of the animals with an average of 1.70 tumors per tumor-bearing animal. When carcinogen-treated animals received dietary colestipol (0.52%, w/w) from 4 weeks prior to the first injection of dimethylhydrazine until the time of death, there was an increase in the number of tumors per tumor-bearing animal to 2.23. In an attempt to understand the nature of this enhancement, animals were administered dietary colestipol at different times in relation to the administration of the carcinogen. The number of tumors per tumor-bearing animal for the different protocols was: post-initiation colestipol exposure, 1.70; colestipol exposure concomitant with dimethylhydrazine, 1.41; pre-initiation colestipol exposure, 2.23.

Thus, colestipol appeared to function both as an anticarcinogen and as a promoter (pre-initiation). Since colestipol has the capacity to bind a number of chemical agents, the different biological effects probably reflect the multifactorial nature of colorectal cancer with the end result dependent on the balance between opposing factors. The selective administration of colestipol in relation to carcinogen administration may prove useful in elucidating the various factors involved in the etiology of this disease.

Introduction

Cancer of the colon and rectum is the most common visceral neoplasm in the United States. Silverberg (*1*) has estimated that approximately 120,000 new cases of colorectal cancer will be diagnosed and about 50,000 people will die of this neoplasm this year.

The etiology of human colorectal cancer is not well understood. Although certain conditions and genetic factors are known to predispose to colorectal cancer, the greatest emphasis in the pathogenesis of this disease has been placed on environmental factors. The importance of dietary factors has been emphasized from epidemiological observations (geographic correlations, migrant studies, low-risk populations, dietary modification trials), and intensive clinical and experimental studies have investigated the role of such factors in the etiology of colorectal cancer. The high incidence of cancer of the colon and rectum in Western countries has been speculatively related to the consumption of inadequate quantities of fiber by Burkitt (*2*), to the consumption of excess of fat and beef protein by Wynder (*3*), and to low amounts of vegetable consumption by Correa (*4*). These food items are not believed to be carcinogenic from the standpoint of initiation, but are viewed as promoting agents in which they, in some way, provide a more favorable environment for initiating carcinogens.

The reported carcinogenic nature of dimethylhydrazine (DMH) by Druckrey et al. (*5*) has led to numerous studies in investigating the carcinogenic process of colorectal cancer. Although epidemiologi-

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cal observations do not establish a causative relationship between dietary factors and cancer, numerous experimental animal studies, as reviewed by Reddy et al. (6) have shown that high fat and protein diets tend to promote DMH-induced colon carcinogenesis.

The experimental protocol usually involves weekly injections of DMH for 5-6 months or longer, and tumors are frequently induced at sites other than the large intestine. A treatment period of 10 weeks was reported by Clapp et al. (7) to produce primarily tumors of the large intestine with few transitional lesions. This schedule was attractive from the standpoint of studies on the modification of carcinogenesis since either an increase or a decrease in tumor incidence could be observed.

Colestipol hydrochloride (Colestid; COL) is a high molecular weight anion exchange resin. It is a copolymer of diethylenetriamine and 1-chloro-2,3-epoxypropane, with approximately one out of five amine nitrogens protonated (chloride form). Colestipol was shown by Stanley et al. (8) to increase the fecal excretion of cholate about 5-fold in healthy volunteers, and similar findings were reported by Rubulis et al. (9) and Grundy and Mok (10). The ability of COL to bind bile acids *in vitro* was demonstrated by Kritchevsky and Story (11, 12). The complex between COL and bile acids is excreted in the feces, thereby resulting in partial removal of bile acids from the enterohepatic circulation. Colestipol is used clinically as adjunctive therapy to treat patients with primary hypercholesterolemia (elevated low density lipoprotein). In a review of the clinical use of COL, Heel et al. (13) reported that the increased fecal loss of bile acids leads to an increased oxidation of cholesterol to bile acids, a decreased level of low density lipoproteins in the serum and a decreased level of serum cholesterol.

The pharmacological properties of COL appear similar to those of another bile acid sequestrant, cholestyramine (Questran; CTY). The addition of dietary CTY (2%, *w/w*) was reported by Nigro et al. (14) and Asano et al. (15) to increase the incidence of intestinal tumors induced by DMH and related agents in rats. In another study by Nigro et al. (16), CTY and candicidin (another serum cholesterol-lowering agent) increased the incidence of intestinal tumors induced by azoxymethane. However, the distribution of the increased number of tumors was different for the two agents. Aluminium hydroxide (Aludrox), a widely used antacid with incidental bile acid-binding properties, was initially reported by Cruse et al. (17) to increase intestinal cancer induced by DMH in rats. However, a subsequent study by Cruse et al. (18) failed to demonstrate any enhancement of DMH-induced intestinal tumors.

It is of interest that MacGregor (19) reported the case of a male who died of cancer of the small intestine following an 8-year history of CTY use, and Sachatello (20) reported a case of colon cancer in a 29-year old male following a 5-year history of CTY therapy secondary to resection of the terminal ileum for trauma.

Therefore, based on the experimental data and the clinical case reports, a study was undertaken to examine the effect of COL on DMH-induced tumors of the large intestine in mice.

Materials and Methods

Male Swiss mice (ICR/SAF), obtained from our own breeding colony and housed in a clean, conventional room with restricted human access, were provided food (pelleted laboratory chow; Ralston Purina Co., St. Louis, Missouri) and water *ad libitum*. The animals were assigned to one of four treatment groups at 10 weeks of age. The animals were housed five per cage in plastic cages with hardwood bedding (Betta chip) in an air-conditioned room with 12-hr intervals of light and darkness. Fifty-five animals were assigned to each treatment group.

Mice in groups I (control) and II (DMH alone) were maintained on unmodified laboratory chow for life. Mice in group I received weekly injections of 0.9% NaCl (pH 6.5 with sodium bicarbonate and containing 0.001 *M* EDTA) at the same time that mice in group II received weekly subcutaneous interscapular injections of DMH (15 mg/kg body weight); all injections were started when the mice were 14 weeks of age. The DMH (Aldrich Chemical Co., Milwaukee, WI) solution was prepared daily in 0.9% NaCl as described above. Animals in groups III (COL) and IV (DMH plus COL) received a diet containing 0.52% COL for various periods of time. The COL-containing diet was prepared by Ralston Purina Co., and the COL was a gift of Upjohn Co. The administration of DMH to animals in group IV was as described above for animals in group II. Surviving animals were killed by cervical dislocation at 40 weeks of age, 26 weeks after the first DMH injection.

The dose schedule was chosen to provide a quantity of tumors such that either an increase or a decrease in the incidence could be observed. The dose of COL approximates, on a per weight basis, the amount administered to patients, but is about 25% of the level of CTY used in previous studies.

Mice were killed by cervical dislocation and examined grossly. The gastrointestinal tract was inflated with Tellyesniczky's fixative *in situ*, removed from the animal, the intestines cleared with methyl

salicylate, stained with Wright's stain, and examined for tumors with the aid of a dissecting microscope at 3× magnification. The intestines were cleared according to the techniques of Spalteholz (as described by Emmel and Cowdry, (21) and Larsen and Ainsworth (22) as modified by Clapp et al. (7). The clearing procedure allows the tumor nodules to be recognized and differentiated from Peyer's patches. The location of the tumors was noted on a schematic drawing for subsequent identification.

Results

The data in this report involved primary tumors in the large intestine induced by repeated injections of DMH. None of the animals which did not receive DMH, either those on unmodified or those on COL-containing diet, developed tumors anywhere along the gastrointestinal tract. Furthermore, no tumors were observed in the small intestine of those animals treated with DMH. Microscopic examination of the tumors revealed the full spectrum of neoplastic lesions as described by Thurnherr et al. (23) and Deschner (24).

The average body weight of all mice at the beginning of the treatments was 32 g. Thereafter the animals were weighed weekly and the average weight of the members of each group did not differ significantly. There was a slow, progressive weight gain up to 20 weeks after the first DMH injections, reaching an average of 46 g, with a decrease in weight during the last month to an average of 43 g in the DMH-treated animals. The majority of the animals remained in good condition until the last few weeks prior to autopsy. The animals, as a rule, did not develop diarrhea.

In the first experiment the animals were placed on the COL diet at ten weeks of age and remained on such throughout their remaining life. Of the 49 DMH-treated mice on the unmodified diet that survived to autopsy, 88% developed tumors of the large intestine; and 85% of the 46 DMH plus COL animals that survived to autopsy developed tumors (Table 1). However, while the DMH group developed 1.70 tumors per tumor-bearing animal, the DMH plus COL group developed 2.23. Tumors were induced along the length of the large intestine. The tumors in both treatment groups tended to occur most frequently in the distal large intestine (Fig. 1). The majority, or 64%, of the isolated tumors in the DMH group were located in the three cm segment of the large intestine above the anus, while the proximal 3 cm of the colon contained relatively few tumors (18%); the corresponding percentages for the DMH plus the COL group were 69% and 8%, respectively. The DMG group developed 36 tumors in the distal 2 cm of the large intestine, whereas the DMH plus COL group developed 50 tumors.

In the post-initiation experiment, the COL diet was initiated at the termination of the DMH injections. The survival of the animals and the percentage of surviving animals with tumors was essentially the same as when the animals received lifetime COL. However, the number of tumors per tumor-bearing animal was not significantly different among the two groups (Table 2). The animals receiving DMH contained 1.55 tumors per tumor-bearing animal, while the group receiving DMH plus COL contained 1.71. The distribution of the tumors was similar to the previous experiment with 61% of the tumors in both groups occurring within the distal 3 cm of the large intestine (Fig. 2). The DMH group contained 29 tumors in the distal 2 cm of the large intestine, while the DMH plus COL group contained 34.

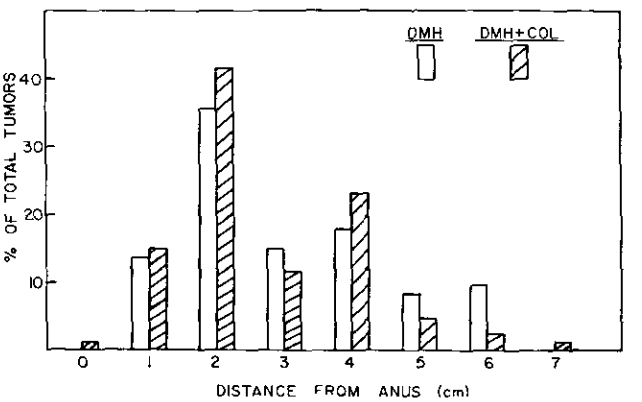

FIGURE 1. Distribution of tumors in the large intestine following dimethylhydrazine and lifetime colestipol.

Table 1. Tumors of the large intestine in mice after dimethylhydrazine administration with or without colestipol.[a]

Treatment group	Number of mice		Number of tumors	
	Surviving	With Tumors	Total	Per tumor-bearing animal
Control	55	0	0	0
DMH	49	43	73	1.70
COL	55	0	0	0
DMH + COL	46	39	87	2.23

[a]Animals received ten weekly injections (between 14 and 24 weeks of age) of dimethylhydrazine (15 mg/kg). Animals were fed the colestipol diet from 10 weeks of age until death.

In another experiment, the animals received dietary COL concomitantly with DMH injections. The fraction of surviving mice with tumors was 84% in the DMH group and 71% in the DMH plus COL group (Table 3). Furthermore, the addition of dietary COL decreased the number of tumors per tumor-bearing animal from 1.79 to 1.41. The relative distribution of tumors in the large intestine was similar to the previous studies (Fig. 3). However, the DMH group developed 40 tumors in the distal 2 cm of the large intestine, and the DMH plus COL group developed only 23.

In the last experiment the COL diet was administered for four weeks prior to the DMH injections. The formation of tumors was increased from 1.64 to 2.23 tumors per tumor-bearing animal (Table 4). The relative distribution of tumors (Fig. 4) did not change significantly. However, the number of tumors in the distal 2 cm of the large intestine increased from 41 in the DMH group to 66 in the DMH plus COL group.

Discussion

In the experiments reported in this paper, tumors of the large intestine were never observed in non-DMH-treated animals. The noncarcinogenic nature of COL observed in mice under the conditions used in these experiments confirmed a previous report by Webster and Bollert (*25*) on the lack of carcinogenicity of COL in rats and dogs.

When the results of the four separate experiments were combined, the schedule of DMH injections used in these studies produced tumors in $88.8 \pm 4.0\%$ (range: 84-94%) of the animals within 26 weeks from the initial DMH injection, and resulted in 1.67 ± 0.10 tumors per tumor-bearing animal (range: 1.55-1.79). The tumors arose primarily

Table 2. Tumors of the large intestine of mice after dimethylhydrazine administration followed by colestipol.[a]

	Number of mice		Number of tumors	
Treatment group	Surviving	With Tumors	Total	Per tumor-bearing animal
Control	55	0	0	0
DMH	45	40	62	1.55
COL	55	0	0	0
DMH + COL	46	42	72	1.71

[a]Animals received ten weekly injections (between 14 and 24 weeks of age) of dimethylhydrazine (15 mg/kg). Animals were fed the colestipol diet from 24 weeks of age until death.

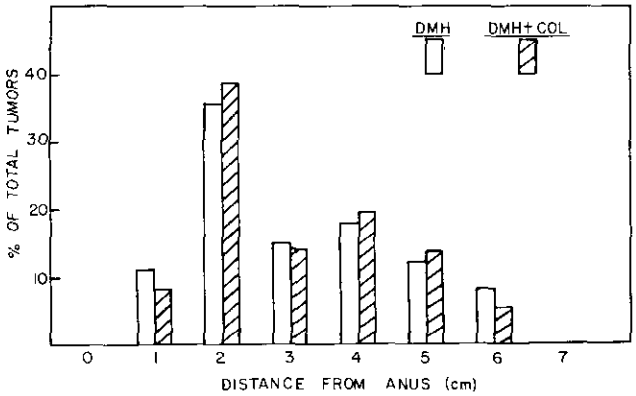

FIGURE 2. Distribution of tumors in the large intestine following dimethylhydrazine and post-initiation colestipol.

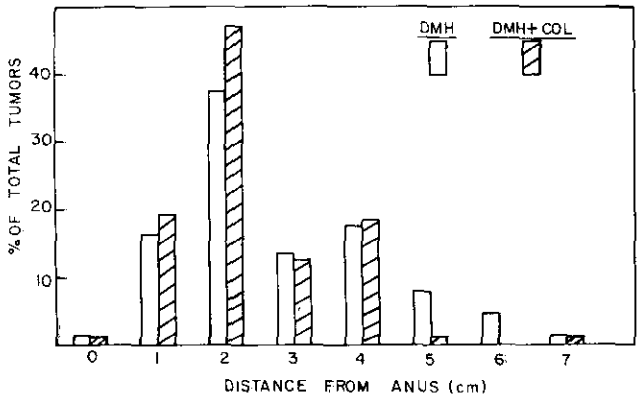

FIGURE 3. Distribution of tumors in the large intestine following dimethylhydrazine with concomitant colestipol.

Table 3. Tumors of the large intestine in mice after dimethylhydrazine administration with concomitant colestipol.[a]

	Number of mice		Number of tumors	
Treatment group	Surviving	With Tumors	Total	Per tumor-bearing animal
Control	55	0	0	0
DMH	50	42	75	1.79
COL	55	0	0	0
DMH + COL	52	37	52	1.41

[a]Animals received ten weekly injections (between 14 and 24 weeks of age) of dimethylhydrazine (15 mg/kg). Animals were fed the colestipol diet during the time they received the injections of dimethylhydrazine.

in the distal portion of the large intestine and were usually isolated.

The initial experiment was conducted with animals which received dietary COL during the last 75% of their life. Since a significant increase in the incidence of DMH-induced tumors was found, additional experiments were designed to determine at what stage in the carcinogenic process COL was producing its effects. The results indicated that COL was not behaving as a promotoer in the classical sense of post-initiation promotion. Rather, COL was found to be a pre-initiation promoter. Furthermore, when COL was administered concomitantly with the carcinogen, it behaved as an anticarcinogen.

The mechanism by which COL influenced DMH-induced tumorigenesis is unknown. It is apparent that any attempts to develop a mechanism of action must approach the problem from the point of view of a multistage/multifactorial process of DMH-induced carcinogenesis. Numerous studies, as reviewed by Maskens (*26*), indicate that DMH is metabolically activated to an electrophilic compound capable of alkylating macromolecules in colon epithelial cells. Thus, each injection of DMH is capable of producing permanent and transmissible changes in some cells that will eventually lead to cancer. However, numerous factors may play a role in modifying the carcinogenic process. These modifying agents may have an additive, synergistic, or inhibitory effect on the activity of a given carcinogen. The observed biological effects of COL on DMH-induced carcinogenesis are probably related to the influence of COL on more than one factor, and some insight into future studies can be ascertained by reviewing some of the factors known to promote or inhibit DMH-induced cancer of the large intestine.

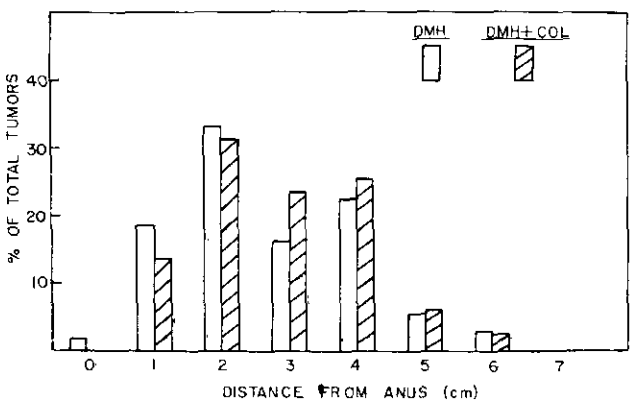

FIGURE 4. Distribution of tumors of the large intestine following dimethylhydrazine and pre-initiation colestipol.

Since COL is a bile acid-sequestering agent, it is important to examine the relationship of bile acids to cancer of the large intestine. Cholesterol is the major, and probably sole precursor of bile acids. During normal digestion bile acids are secreted into the intestines via the bile from the liver and gallbladder. Bile acids emulsify the fat and lipid materials in food, thus facilitating absorption. A major portion of the bile acids that are secreted are reabsorbed from the intestines and returned via the portal circulation to the liver, thus completing the enterohepatic cycle. The decrease in serum cholesterol levels as a result of COL therapy is due to sequestering bile acids which result in an increased fecal excretion of bile acids and interference with their normal enterohepatic circulation. As a result there is a compensatory increase in cholesterol catabolism to form additional bile acids along with an increased synthesis of cholesterol in the liver.

Evidence for the importance of bile acids as cancer promoters in the large intestine has developed from studies by Chomchai et al. (*27*), Narisawa et al. (*28*), Reddy et. al. (*29-31*), and others. The mechanism of action of tumor promotion by bile acids is unknown. However, it is of interest that Kulkarni et al. (*32*) reported that lithocholic acid produced single-strand breaks in the DNA of L1210 leukemia cells, and Kulkarni and Yielding (*33*) reported single-strand breaks in DNA from crypt cells of mouse colon induced by lithocholic acid.

Thus, the anticarcinogenic effect of COL, observed when COL was administered simultaneously with DMH, can be rationalized on the basis of COL binding the bile acids in the intestinal lumen and preventing the interaction of these tumor promoters with colonic mucosa. However, when animals were given dietary COL before, during and after DMH, or merely before the carcinogen, there was

Table 4. Tumors of the large intestine in mice after dimethylhydrazine administration following colestipol.[a]

Treatment group	Number of mice		Number of tumors	
	Surviving	With Tumors	Total	Per tumor-bearing animal
Control	55	0	0	0
DMH	48	45	74	1.64
COL	55	0	0	0
DMH + COL	46	44	98	2.23

[a]Animals received ten weekly injections (between 14 and 24 weeks of age) of dimethylhydrazine (15 mg/kg). Animals were fed the colestipol diet for 4 weeks prior to the injections of dimethylhydrazine.

an increased incidence of tumors in the large intestine. The observed duality of effects due to COL is similar to those reported for butylated hydroxytoluene (BHT). When BHT was given prior to and concurrently with 2-acetylaminofluorene, as reported by Ulland et al. (*34*), or azoxymethane, as reported by Weisburger et al. (*35*) it protected against carcinogenesis. However, Peraino et al. (*36*) and Weisburger et al. (*35*) demonstrated a promoter activity for BHT when given after 2-acetylaminofluorene or azoxymethane, respectively.

Conflicting results are frequently reported when investigating the effects of bile acid binding agents on carcinogenesis of the large intestine. Nigro et al. (*14, 16*) and Asano et al. (*15*) reported that the coadministration of CTY at a level of 2% (*w/w*) increased the incidence of tumors. Aluminum hydroxide was initially reported by Cruse et al. (*17*) to enhance DMH-induced carcinogenesis, but no promoter activity was found in a latter study by Cruse et al. (*18*).

The effect of bran, which binds bile acids to a lesser degree than CTY or COL, has been widely studied since the epidemiological studies by Burkitt (*2*) implicated low-fiber diets as a factor in the etiology of human colorectal cancer. However, the findings of Draser and Irving (*37*), Hill (*38, 39*), and Eastwood et al. (*40*) have challenged these findings. It has been argued that dietary fiber would be anticarcinogenic in that it would increase fecal output and decrease intestinal transit time. Barbolt and Abraham (*41*) reported bran decreased carcinogen-induced cancer, Cruse et al. (*42*) reported that bran had no effect on the incidence of cancer, and Clapp et al. (*43*) presented results that showed bran to be a tumor promoter. The discrepancies in the results of experimental systems could be due to differences in the fiber components, differences in nonfiber components, absorption of other factors by the fiber, and different effects on the intestinal flora. The variability of dietary fiber in animal diets and the relevance of this variability to experimental studies has recently been discussed by Wise and Gilburt (*44*).

The observed promoter activity of bile acid-binding agents is usually explained on the basis that the binding to bile acids results in an increased delivery of these tumor promoters to the large intestine. However, these bile acids are sequestered in the resin matrix, and this explanation may be insufficient to account for the promoter activity. Indeed, it is difficult to explain both the anticarcinogenic and promoter activity of COL based on its interactions with bile acids. Much work is obviously required to unravel the complex interrelationships between bile acids, bile acid-sequestering agents and cancer of the large intestine. The nonselective nature of binding as well as variability in composition and purity of bile acid binding agents serves to further emphasize the need for additional experimental work in this area.

It is well known that COL and CTY are nonspecific anion-exchange resins; their binding capability is not restricted to bile acids, but includes other acidic drugs and biomolecules. The chronic use of COL was reported by Miller et al. (*45*) to be associated with an increased bleeding tendency due to hypoprothrombinemia from vitamin K deficiency, and Ko and Royer (*46*) reported that warfarin and tetracycline were significantly bound by COL. A large portion of vitamin B_{12}-intrinsic factor complex, folic acid, and iron citrate was reported by Leonard et al. (*47*) to be bound by COL and CTY.

In view of the binding of vitamin B_{12}-intrinsic factor complex by CTY and COL, it is of interest that Yamamoto (*48*) reported that vitamin B_{12} was necessary for the formation of DMH-induced tumors. Studies by Cook and McNamara (*49*) have suggested the involvement of vitamin E in colorectal cancer.

Vahouny et al. (*50*) demonstrated that commercial ion-exchange resins and certain dietary fibers sequester phospholipids from micellar media. The effect of these agents on intestinal morphology and carcinogen-induced tumors is unknown. Vahouny et al. (*51*) presented evidence that bile acid sequestering agents induce morphological changes in the intestines, and previous studies by Cassidy (*52, 53*) had reported similar findings. It was suggested that the alterations induced in intestinal morphology may provide a means of increasing the sensitivity of the colon to chemical carcinogens.

The promoting activity of nonspecific injury was demonstrated by Rous and Kidd (*54*), and the tumor-promoting effect of tissue injury on DMH-induced carcinogenesis was described by Pozharisski (*55*). A clustering of experimental tumors around suture lines or colostomy stomas has been reported by Witting (*56*) and Harte et al. (*57*) and others, and human colon cancers have been reported by Floyd et al. (*58*) to occur with increased frequency at colonic suture lines. Mueller and Thornbury (*59*) described colon cancer as a complication of ureterosigmoidostomy.

Major small bowel resection was shown by Williamson et al. (*60, 61*) to result in long-term adaptive mucosal hyperplasia in the remaining bowel. When a single initiating dose of DMH was injected into rats, followed by resection of the distal third of the small intestine, Tilson (*62*) reported an increase in the number of tumors. Celik et al. (*63*) observed an increased incidence of tumors in the dilated splenic flexure following jejunocolic transposition and sug-

gested that the observed chronic irritation and inflammation may play a role in this effect.

It is tempting to speculate that the pre-initiation promotion observed with COL in the studies reported in this paper may be due to the increased sensitivity of the colonic mucosal cells to DMH following four weeks of exposure to COL.

Another possible factor which may be influenced by COL is the immune system. There is increasing evidence that endogenous and exogenous lipids are involved in some way in modulating or regulating the immune system. The recent reports, as reviewed by Broitman (*64*), on the association of lowered cholesterol levels with increased cancer of the large intestine, is of interest. The available evidence, as reviewed by Posner et al. (*65*) suggests that dietary manipulation which results in tumor promotion also results in immunosuppression.

In addition, low density lipoprotein was reported by Morse et al. (*66*) to inhibit lymphocyte proliferation. The binding of low density lipoprotein by human fibroblasts and lymphocytes modulates the rate of uptake, esterification, and synthesis of cholesterol as reported by Curtis and Edington (*67*) and Ho et al. (*68*). Vitale and Broitman (*69*) suggested that the changes in the concentration of lipoproteins may enhance the growth of premalignant cells. Since COL alters the concentration of low density lipoprotein it is possible that the promotion effects of this agent are related to the lipoproteins.

Monocytes and macrophages are involved in regulating the immune response. These cells may function as suppressor cells, exerting their suppressor effect on other lymphoid cells by secreting prostaglandins. Balch and Tilden (*70*) reported that prostaglandin-producing suppressor cells were involved in the decreased lymphocyte function observed in some cancer patients, and the decreased response to mitogens could be restored by incubation with indomethacin.

Jaffe (*71*) Bennett and Del Tacca (*72*) Cumming and Robertson (*73*) and Bennett et al. (*74*) have described the production of prostaglandins by various human and animal tumors. The growth of transplantable tumors was reported by Sykes and Maddox (*75*) Levine et al. (*76*) and Lynch and Salomon (*77*) to be inhibited by prostaglandin inhibitors. Pollard and Luckert (*78*) observed that indomethacin reduced the incidence and frequency of DMH-induced tumors in the rat colon. The effect of COL on prostaglandins in unknown.

Although the precise mechanism for either the anticarcinogenic or promoter effect of COL remains to be elucidated, the effects are probably indirect since COL is not absorbed from the gastrointestinal tract. The multifactorial nature of carcinogenesis suggests that the effects of COL are related to: (a) the bile acid sequestering activity of this agent; (b) alterations in absorptive or morphological properties of tissues in such a way that the carcinogenicity of DMH is enhanced; (c) the binding to other biomolecules which may be involved in malignant transformation either as agents of promotion or inhibition; (d) modification of immunological processes; and/or (e) alterations in the intestinal flora. It is apparent that COL alters opposing aspects of the carcinogenic process, that is, protective and predisposing. The end result depends on the balance between opposing factors. Current studies are underway to examine the role of these factors in the modification of DMH-induced cancer by COL.

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